

Protocol for the Determination of Optimal Concentration of DEAE-Dextran

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I. Introduction

Serum and plasma samples are tested for the presence of neutralizing antibodies by using specific assays that are described in various Protocols (Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells; Neutralizing Antibody Screening Assay for HIV-1 in TZM-bl Cells) and use supporting procedures as described in various Protocols (Heat-inactivation of Serum and Plasma Samples; Trypsin-EDTA Treatment for Disruption of Cell Monolayers; Preparation and Titration of HIV-1 Env-pseudotyped Viruses and Preparation and Titration of HIV-1 IMC Viruses).

In order to achieve optimal levels of virus/pseudovirus infection, it is recommended to supplement the assay medium with DEAE-Dextran. This polycation counters the repulsive electrostatic forces between the virus and cells surface without affecting antibody binding and neutralization. However, DEAE-Dextran from different sources and different lots may exhibit substantial variability in potency and cell toxicity. For this reason, each new batch of DEAE-Dextran, regardless of lot, should be titrated by performing serial dilutions in a 96-well plate in each respective assay above. The optimal concentration of DEAE-Dextran is determined from the dilution that yields the highest relative luminescence units (RLU) and has no detrimental effects on the cell as observed by light microscopy after incubation.

II. Definitions

DEAE-Dextran:	Diethylaminoethyl-Dextran
GM:	Growth Medium
Luc:	Luciferase
RLU:	Relative Luminescence Unit
DPBS:	Dulbecco's Phosphate Buffered Saline
TCID:	Tissue Culture Infectious Dose
IMC:	Infectious Molecular Clone
COA:	Certificate of Analysis

III. Reagents and Materials

Recommended vendors are listed. Unless otherwise specified, products of equal or better quality than the recommended ones can be used whenever necessary.

General reagents and materials are listed in the Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells

IV. Instrumentation

Recommended manufacturers are listed. Unless otherwise specified, equipment of equal or better quality than the recommended ones can be used whenever necessary.

Equipment required for this protocol are listed in the Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells.

V. Specimens

Not Applicable.

VI. Protocol

1. Criteria for Deciding When the Optimal DEAE-Dextran Concentration Needs to be Determined

- 1.1. The optimal concentration of DEAE-Dextran for use in neutralization assays should be determined via a toxicity test each time a new batch of DEAE-Dextran is prepared (Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells), regardless of lot or receipt information.

2. Titration of DEAE-Dextran in TZM-bl Cells

NOTE 1: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

- 2.1. Using the format of a 96-well flat bottom culture plate as illustrated in Figure 1, place 40 µl of growth medium (GM) in all wells in the entire plate. Place an additional 152 µl of GM in all wells of column 1 (to receive DEAE-Dextran). Place an additional 50 µl to column 12 (cell control).
- 2.2. Add 8 µl of test DEAE-Dextran (5 mg/ml stock solution) to each well in column 1 (rows A-H). Mix the samples in column 1 and transfer 160 µl to column 2. Repeat the transfer and dilution of DEAE-Dextran through column 11 (these are serial 1.25-fold dilutions). After the final transfer and mixing is complete, discard 160 µl from the wells in column 11 (rows A-H) into waste container. Wells in column 12 will serve as cell controls for background luminescence (no virus added).

NOTE 2: This format is designed to measure DEAE-Dextran concentrations in the range of 32 µg/ml down to 3.4 µg/ml. Appropriate adjustments may be made to test a different range of dilutions. Previous validation experiments have shown that the possible DEAE-Dextran concentration optimal for use in the neutralization assay is between 30 µg/ml and 7.5 µg/ml. This format is designed to assay two viruses/pseudoviruses in quadruplicate wells at each DEAE-Dextran concentration per plate (Figure 1).

- 2.3. Thaw the required number of vials of each virus/pseudovirus by placing in an ambient temperature water bath. When completely thawed, appropriately dilute each virus/pseudovirus

in GM in two separate reservoirs. (See Protocol for Preparation and Titration of HIV-1 Env-pseudotyped Viruses for measurement of TCID and selection of virus dose.)

- 2.4. Dispense 50 µl of the first virus/pseudovirus to all wells in columns 1-11, rows A through D.
- 2.5. Dispense 50 µl of the second virus/pseudovirus to all wells in columns 1-11, rows E through H.
- 2.6. Dispense 160 µl of prepared TZM-bl cell suspension (10,000 cells per well) to each well in columns 1-12, rows A through H.

NOTE 3: To minimize carry over, always add cells and pseudovirus from the column that contains the smallest concentration of DEAE-Dextran and proceed to the column that contains the greatest concentration of DEAE-Dextran.

- 2.7. Cover the plate and incubate for 48 hours.

NOTE 4: Examine all wells for normal cell morphology and viability by microscopic examination. It is important to note the presence of unhealthy cells and/or toxicity as certain doses of DEAE-Dextran can cause detrimental effects to the cells and thus the validity of assays will be compromised. If cell stress and/or toxicity are present at any given concentration, this particular dose of DEAE-Dextran should not be used in the assays.

- 2.8. For TZM-bl cell assays with pseudovirus using Britelite Plus or Bright-Glo

- 2.8.1. Remove 150 µl of culture medium from each well, leaving approximately 100 µl.
- 2.8.2. Dispense 100 µl of BritelitePlus or Bright-Glo Reagent to each well.
- 2.8.3. Incubate at room temperature for 2 minutes to allow complete cell lysis. Mix by pipettor action (at least two strokes) and transfer 150 µl to a corresponding 96-well black plate. Read the plate immediately in a luminometer.

3. Determination of Optimal DEAE-Dextran Concentration

- 3.1. The optimal concentration of DEAE-Dextran is determined from the dilution that yields the highest RLU and has no detrimental effects on the cells as observed by light microscopy after the respective incubation time period.

NOTE 5: If the optimal DEAE-Dextran concentration is 10 µg/ml in an assay plate, use 50 µl of the 5 mg/ml stock solution per one neutralization assay plate (DEAE-Dextran concentration in the cell suspension is 25 µg/ml). For TCID assays, use 40 µl of 5 mg/ml stock solution per one TCID plate (DEAE-Dextran concentration in the cell suspension is 20 µg/ml).

4. Testing the newly determined optimal DEAE-Dextran concentration

- 4.1 After determining the optimal concentration of DEAE-Dextran, test the dilution in a neutralization assays test plate before distributing the reagent.
- 4.2 Using the format of a 96-well flat bottom culture plate, place 150 µl of the appropriate GM in all wells of column 1 (cell control). Place 100 µl in all wells of columns 2-12.

- 4.3** Thaw the required number of vials of each virus/pseudovirus (optimally the same viruses used for the DEAE-Dextran titration) by placing in an ambient temperature water bath. When completely thawed, appropriately dilute each virus/pseudovirus in GM in two different reservoirs. (See protocols for Preparation and Titration of HIV-1 pseudotyped-Viruses and Env.IMC.LucR Viruses for measurement of TCID and selection of virus dose).
- 4.4** Dispense 50 µl of the first virus/pseudovirus (optimally the same viruses used for the DEAE-Dextran titration) to all wells in columns 2-12 (one virus/pseudovirus tested per plate) as described in Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells.
- 4.5** Cover the plates and incubate for 45-90 minutes.
- 4.6** During the incubation, prepare a suspension of cells using the newly determined optimal concentration of DEAE-Dextran as described in Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells.
- 4.7** Dispense 100 µl of the prepared cell suspension to each well in columns 1-12, rows A-H.
- 4.8** Cover the plates and incubate for the amount of time as described in Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells.
- 4.9** After incubation, observe by light microscopy at least 10 wells that contain virus and cells for detrimental effects. Choose a lower dilution of DEAE-Dextran or repeat the titration if cells are not healthy.

Figure 1: Assay template for measuring cell toxicity via titration of DEAE-Dextran, 2 viruses per plate

Virus One												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
B	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
C	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
D	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
E	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
F	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
G	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
H	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
32µg/ml 25.6µg/ml 20.5µg/ml 10.4µg/ml 3.1µg/ml 10.5µg/ml 8.4µg/ml 6.7µg/ml 5.4µg/ml 4.3µg/ml 3.4µg/ml 0.0 µg/ml												

Virus Two

The concentrations listed below the table are the final concentrations of DEAE-Dextran in each well.
CC, Cell control wells (cells only).